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ORIGIN OF MURINE B CELL LINEAGES

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Abstract

Until recently, the hematopoietic stem cells (HSC) that appear early in ontogeny were thought to constitute a homogeneous, self-replenishing population whose developmental potential remains constant throughout the life of the animal. Studies reviewed here, however, demonstrated clear differences in the developmental potential of fetal and adult progenitor populations (including FACS-sorted HSC). These studies, which chart the ability of various progenitor sources to reconstitute functionally distinct B cell populations, define three B cell lineages: B-1a cells (CD5 B cells), derived from progenitors that are present in fetal omentum and fetal liver but are largely absent from adult bone marrow; B-1b cells ("sister" population), derived from progenitors that are present in fetal omentum, fetal liver, and also in adult bone marrow; and conventional B cells, whose progenitors are missing from fetal omentum but are found in fetal liver and adult bone marrow. B-1a and B-1b cells share many properties, including self-replenishment and feedback regulation of development. These B cell studies, in conjunction with evidence for a similar developmental switch for T cells and erythrocytes, suggest that evolution has created a "layered" immune system in which successive progenitors (HSC) reach predominance during development and give rise to differentiated cells (B, T, etc) responsible for progressively more complex immune functions.

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INTRODUCTION

B cell populations are distinguishable by an ensemble of properties. No single characteristic distinguishes any population; however, sets of characteristics allow the clear recognition of several populations of cells, all of which share a commitment to the production of immunoglobulin. These populations can be recognized on the basis of differentiation status (e.g. pre-B cells, resting B cells, plasma cells), anatomical localization (e.g. marginal zone B cells, follicular B cells, peritoneal B cell), surface phenotype (e.g. IgM and IgD levels, presence of CD5, presence of MAC-1) or, our focus here, the progenitors from which they arise and hence the developmental lineage to which they belong (e.g. B-1 cells formerly known as Ly-1 B cells (1), conventional B cells).

Over the years, several B cell lineages have been proposed. Wortis suggested that the B cells in nude mice and the B cells found in xid mice belong to different developmental lineages (2), and MacLennan and colleagues proposed that splenic marginal zone B cells are a distinct lineage (3, 4). Recently, Linton et al described two precursor populations in the spleen that mostly yield either primary antibody forming cells (AFC) ($J11D^{hi}$) or secondary AFC ($J11D^{lo}$) (5). Finally, our laboratory and others have identified a population of $CD5^+$ B cells in the peritoneal cavity that several laboratories have now collectively shown to belong to a distinct developmental lineage. In this review, we focus on the evidence underlying this major lineage distinction (i.e. B-1 vs conventional B cells) and the substantial evidence for distinct progenitors that has accrued.

Definition of a Developmental Lineage

Webster's Dictionary defines lineage as "descent in a line from a common progenitor" (6). Developmental biologists adhere to this definition; however, there is often considerable discussion, particularly with respect to the immune system, as to what characteristics define a lineage and its progenitor. This definition is often made on practical grounds: in the broadest sense, all cells in a given animal can be assigned to a single lineage, since the zygote is the ultimate progenitor; at the other extreme, the progeny of a single, newly arisen B cell can be treated as a lineage because such B cells are distinguished from each other by unique immunoglobulin rearrangements. By and large, however, developmental lineages are defined as deriving from relatively undifferentiated progenitors that have at least a limited capacity for self-renewal, and they give rise to progeny that are committed to differentiate into cells with particular functional characteristics.

Originally, a single hematopoietic stem cell (HSC) was thought to be

the progenitor of all cells in the hematopoietic system. This stem cell was recognized in early fetal tissue and in adult bone marrow and spleen by its ability to reconstitute an apparently normal hematopoietic system in irradiated recipients (for review, see 7–9). In essence, viewed with the methodology available at the time, the lymphoid, erythroid, and myeloid cells regenerated from either the fetal or the adult HSC appeared identical. Thus, the HSC was assumed to perpetuate itself without change.

Evidence potentially inconsistent with this view has begun to accumulate. For example, studies of erythroid differentiation in the sheep indicate that the early fetal HSC are committed to giving rise to fetal erythrocytes, which express $\alpha_2\gamma_2$ hemoglobin, whereas more mature HSC are committed to generating erythrocytes of the adult phenotype ($\alpha_2\beta_2$ hemoglobin) (10–13). More recently, similar reconstitution studies showed that HSC in adult bone marrow fail to regenerate murine $CD5^+$ B cells which were newly identified by multiparameter FACS analyses (14). Nevertheless, the idea that fetal HSC perpetuate themselves without changing throughout adulthood dominated immunological thinking until recently, when compelling evidence demonstrated differences between the reconstitution potential of fetal and adult HSC.

Data showing that progenitors found at different times during ontogeny are committed (programmed) to differentiate into particular lymphocyte populations come from separate studies of B and T cell development. This review focuses on the B cell studies, which raised the initial challenge to the "single progenitor" hypothesis for lymphocytes and which have now provided definitive evidence for distinctive progenitors for B cell subsets and hence for distinctive B cell lineages. However, similar arguments can be made for T cells. For example, Ikuta and coworkers have dramatically demonstrated that $V\gamma3$ T cells, which are the first T cells to develop in the thymus, (15–17) can develop from fetal HSC but not adult bone marrow HSC (6, 18). Taken together, these findings concerning the origins of lymphocyte subsets force the enlargement of the older paradigm to allow for changes in the potential of the HSC that function at different times during development.

DISTINGUISHING B CELL SUBSETS

In this section, we summarize the properties of the B cell lineages. Because we are primarily interested here in the ontogeny of these lineages, we focus mainly on those whose origins have been extensively investigated. Thus, we treat "conventional" B cells (which include almost all of the B cells in lymph node and spleen) as a single entity even though subdivisions that may reflect additional lineage distinctions have been described. In contrast,

although the B–1 cell population (1), which is concentrated in extra-lymphoid sites such as the peritoneal and pleural cavities, is substantially smaller, we subdivide it into two separate populations, B–1a cells (CD5$^+$ or Ly–1$^+$ B cells) and B–1b cells (CD5$^-$ Ly–1 B sister population). This subdivision is consistent with evidence (discussed later) indicating that these two quite similar cell types arise from separate progenitors and thus represent separate lineages. Several other reviews that focus on the phenotype, repertoire, and functions of B–1 cells, including the homologous human population, are available (19–26).

Cell Surface Phenotype

The introduction of multiparameter FACS analysis has facilitated the characterization of B cell populations on the basis of cell surface antigen expression and size (27). This method led to the identification of murine B cell tumors that express Ly–1 (CD5), a cell surface glycoprotein which previously had been thought to exist only on T cells (28, 29). Subsequently, a subpopulation of splenic B cells that express IgM and IgD was shown also to express low to moderate levels of Ly–1 (30–32). Other markers have been used to further characterize the differences between the Ly–1 B cells, which we now call B–1a cells (1): conventional B cells, also known as B–2 cells, and other B cell populations. Peritoneal B cell populations are identified by FACS in Figure 1, and Table 1 summarizes some of the differences in cell surface phenotype for B–1 and conventional B cells.

Two markers deserve special attention. Mac 1(CD11b) is present on peritoneal and pleural cavity B–1 cells but is not expressed on either conventional B cells or splenic B–1 cells. FcεR (CD23) is present on all conventional B cells in the peritoneal cavity and on the predominant

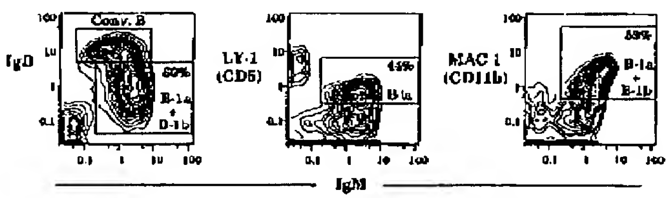

Figure 1 FACS analysis of peritoneal B cell populations. Conventional B cells are identified by a broad, positive IgM and tight, bright IgD FACS profile. They are negative for CD5 (Ly-1) and Mac 1. All B–1 cells are IgM bright and low to moderate for IgD. B–1 cells are also MAC 1 positive in the peritoneum. B–1 cells are divided into B–1a cells which are CD5$^+$ and B-1b cells which are CD5$^-$. The number of B–1b cells is obtained from the difference of total B–1 cells and B–1a cells. Direct gating on CD5$^-$ IgM^{hi} cells is avoided because of overlap with conventional B cells. Plots are 5% probability contours, generated with gating for live lymphocytes by forward and obtuse scatter and propidium iodide.

Table 1 Selected markers on B–1 and conventional B cells. References (27, 30, 31, 33–35, 41, 43, 154), references therein and unpublished observations. Here, conventional B cells do not include marginal zone B cell which are IgD^{lo} and FcεR$^-$

Marker	B-1 cells	Conventional B cells
IgM	+++	+
IgD	+/− to ++	+++
CD5 (Ly-1)	+ on B-1a, − on B-1b	−
CD11b (MAC 1)	+ in PerC, − in Spleen	−
CD23 (FcεR)	−	+
B220 (RA3-6B2)	++	+++
B220 (other)	+	+
IL5R	+	some, inducible
CD72	+	++

(IgD^{hi}) conventional B cell population in the spleen; however, it is not expressed on either marginal zone (IgD^{lo}) B cells in the spleen or B–1 cells from any location. Thus, in the peritoneal cavity (but not in the spleen), these markers alone can be used to distinguish B–1 cells from conventional B cells, i.e. B–1 cells are Mac1$^+$ and FcεR$^-$ whereas conventional B cells are Mac1$^-$ and FcεR$^+$ (33–35).

ACTIVATION MARKERS Several other markers can be used to distinguish B-1 cells from the typical "resting" conventional B cells (IgD^{hi} FcεR$^+$) that predominate in spleen and lymph node. Some of these, however, are also expressed on some types of "activated" conventional B cells. This has raised questions about the activation status of the B–1 population.

The definition of an activated B cell is necessarily vague since the activation protocols can engender different differentiation states that express overlapping but distinct subsets of markers. For example, there is no difference in the level of IL–2R or transferrin receptor expression, presumed markers of intermediate B cell activation, on CD23$^+$ (conventional B cells) and CD23$^-$ (marginal zone + B–1 cells) splenic B cells (36). The majority of both populations are also negative for S7 (CD43), a marker reported to be present on B cells undergoing terminal B cell differentiation (36, 37). However, further studies with S7 reveal that many splenic and peritoneal B–1 cells express the marker, including those cells that secrete antibody (S. M. Wells, A. B. Kantor, A. M. Stall, in preparation). LPS stimulation, which in vivo leads readily to IgM secretion and the development of IgM-secreting plasma cells, induces BLA–1 and BLA–2 expression, but not CD5 on splenic B cells (38). In contrast, Ying-zi et al have shown that CD5, as well as some other markers associated with the B–1a phenotype, can be induced on splenic conventional B cells

following in vitro stimulation with mitogenic anti-μ plus IL-6 (39). The authors suggest that their in vitro stimulation with anti-μ is a model for in vivo T-independent (type 2, TI-2) stimulation which produces B-1a-phenotype cells from conventional (IgD^{hi}) B cells.

However, the B-1a phenotype is not induced with the classic TI-2 antigen, TNP-Ficoll. Hayakawa et al found that the plaque forming cells (PFC) are *not* in the FACS sorted Ly-1 B cell population; <2% of the TNP-PFC were Ly-1$^+$ B cells (105). Anti-bromelein-treated mouse red blood cell (BrMRBC) (105) and antiphosphorylcholine (PC, T15 idiotype) PFC (39a, 115) are found in the Ly-1$^+$ B cell fraction, indicating Ly-1 is indeed retained on B-1a PFC.

Also, contrary to the results of Ying-zi et al, stimulation of splenic B cells with multivalent anti-μ or anti-δ coupled to dextran (both of which are potent TI-2-like antigens and which extensively cross-link sIg and cause proliferation—40) does not induce CD5 expression. Moreover, the IL-5R, which is found on B-1 cells (41, 42), is induced on splenic conventional B cells with the anti-Ig-Dex stimulation (43). The difference between the two protocols may relate to the specific MAbs used and/or the level of endotoxin contamination in the preparation. Thus, there is still much to be learned about the significance of the expression of various "activation" markers and how they relate to the activation state(s) of the cells they mark.

CD5 EXPRESSION: B-1a VS B-1b CELLS Murine B-1 cells were initially identified because they had low but clearly detectable levels of surface CD5. Later, as FACS technology improved and the characteristic phenotype of these cells became more clearly delineated, we recognized a CD5$^-$ B-1 cell subpopulation whose phenotype, localization, functionality, and replenishment characteristics appear to be identical to the CD5$^+$ B-1 cells (33, 34, 44). Reconstitution studies discussed later (see Progenitors) suggest that these two very similar subpopulations represent closely related but distinct B cell lineages that are reconstituted by separate progenitors. We refer to the cells in these populations/lineages as B-1a cells, which do express detectable levels of surface CD5, and B-1b cells, which do not.

The distinction between the B-1a and B-1b lineages is also reflected in the genetically controlled variation in their frequencies in different mouse strains. Thus, there is genetic variation in the number of B-1a cells (21, 29) and in the number of B-1b cells (34) found in different mouse strains. For example, the fraction of PerC B-1 cells that are B-1b is 20-25% in Balb/c congenics and 40-50% in CBA congenics. The RHS/J strain is reported to have low levels of peritoneal B-1a cells, but many B-1b cells (45, 46). Thus far there are no known functional differences between B-1a and B-1b cells. However, the absence of CD5 on B-1b cells and the presence of its ligand CD72 (47, 48) on both B-1a and B-1b cells suggest that such differences will be found.

FACS DETECTION OF B-1 CELLS In principle, the recognition of B-1 cells should be readily achievable in all laboratories that have adequate FACS instruments that are maintained in good condition. In practice, however, certain precautions must be observed (for general reviews, see 49, 50). First, the machine should be standardized before each use, preferably with stable dye-encapsulated polystyrene microspheres, to ensure reproducibility. Second, care should be given to the reagents used: they should be appropriately specific, bright, and titrated to ensure saturating levels without unnecessary background. For example, in the type of B cell transfers discussed here, anti-Ig allotype reagents must not cross-react with the other allotype, and the anti-CD5 reagent must be bright enough to distinguish B-1a and B-1b cells from each other and from T cells. Third, fluorescence compensation for dye overlap should be set properly.

Fourth, for the detection of rare cells, background staining should be minimized and doublets excluded by appropriate counter staining and gating. For example, a doublet containing a CD5$^+$ T cell and an IgM$^+$ B cell, which might be counted as a B-1a cell in the evaluation of thymic T cells, could be avoided by excluding T cells with CD4 and CD8. Dead cells should also be gated out with propidium iodide. Finally, it is advantageous to evaluate particular B cell subsets with multiple markers. For routine analysis of peritoneal B cells we always use IgM, IgD, CD5, and Mac 1, and often use CD23 and B220 (RA3-6B2).

Anatomical Localization

B-1 and conventional B cells can be distinguished by their anatomical localization. B-1 cells develop early in ontogeny and are readily detected in the neonatal spleen (31). In the adult, B-1 cells predominate in the peritoneal and pleural cavities (14, 51, 52) but are rare in lymph node, Peyer's Patches, and peripheral blood. B-1 cells represent a few percent of the total B cells in adult spleen, most of which are conventional B cells. Note, however, that there is an approximately equal number of B-1 cells in the spleen and peritoneum, e.g. $\sim 3 \times 10^6$ in a normal BALB/c adult. The small number of Ig$^+$ B cells detected in thymus cell suspensions has also been reported to be CD5$^+$ (53).

Progeny of B-1 cells are also clearly detectable. B-1 cells give rise to large numbers of Ig-secreting plasma cells. Although there is no known distinguishing phenotype for these cells, they can be identified in Ig-allotype chimeras with appropriately allotype-specific reagents. In particular,

the B-1 population makes a large contribution to the IgA-secreting plasma cells of the intestinal lamina propria and the IgM-secreting cells in the spleen (54-56).

B-1 Cells Are Self-Replenishing

In contrast to conventional B cells, which are replenished throughout life by differentiation of unrearranged progenitors based in the bone marrow, B-1 cells maintain their numbers in adult animals by self-replenishment (14, 57). Both kinds of B cells turn over at the same rate (1% day^{-1}) (58-60). However, virtually no newly differentiated B-1 cells enter the peripheral pool in adults, whereas undifferentiated progenitors in the bone marrow continually give rise to (newly arisen) conventional B cells.

In vivo labeling studies have shown that although bone marrow directly gives rise to splenic B cells, few (~1%) of these newly formed B cells enter the long-term recirculating pool (61). These data mainly reflect the dynamics of the conventional B cell population. Adult bone marrow contains few if any self-replenishing B-1 cells and largely fails to reconstitute B-1 cells (particularly B-1a cells) when it is transferred to irradiated recipients (14, 62, 63). Thus it mainly provides a continuing progenitor source for the replenishment of conventional B cells.

B-1 cells, in fact, neither need nor use a continuing progenitor source in adults. Reconstitution studies show clearly that B-1 cells can maintain their numbers by self-replenishment, i.e. by division of fully mature B-1 cells. FACS-sorted IgM^+, $CD5^+$ B-1a cells completely and permanently reconstitute the B-1a population in transfer recipients (57). Similarly, FACS-sorted B-1b cells completely and permanently reconstitute the B-1b population. Within experimental limits, each sorted B-1 population replenishes itself but not the other (33, 44), indicating that these populations are independently maintained. In addition, in vivo feedback regulation studies (see Feedback) show that the entry of cells into the B-1 pool terminates shortly after weaning, since depletion of a component of the neonatal B-1 population results in the depletion of that component throughout life. Thus, the in situ B-1 population must persist via self-replenishment rather than de novo differentiation.

CAN CONVENTIONAL B CELLS RECONSTITUTE THEMSELVES? The question of whether some conventional B cells also persist via self-replenishment is more difficult to address. Antigenic stimulation induces IgM^+, IgD^+ conventional B cells to differentiate into memory B cells that mainly switch to IgG-expressing cells that persist for the life of the animal. These cells, which may divide infrequently in situ in the absence of antigen, readily reconstitute the memory population in (antigen-stimulated) adoptive

recipients and thus qualify as self-replenishing cells (64). They do not, however, reflect the behavior of typical IgM-bearing conventional B cells, which consistently fail to reconstitute the overall conventional B cell population when FACS-sorted cells are transferred to irradiated recipients.

Small numbers of transferred conventional B cells may persist for many months in adoptive recipients and may even be capable of limited self-replenishment. Careful analysis reveals their presence in appropriate recipients (65) (A. B. Kantor, A. M. Stall, S. Adams, L. A. Herzenberg, in preparation). Their low numbers suggest either that they persist without being able to divide at a rate sufficient to replenish the overall conventional B cell population, or that they represent a unique subset of self-replenishing conventional B cells.

Because the issue of the persistence of these transferred conventional B cells has raised questions concerning the difference in potential for self-replenishment between conventional and B-1 cells (65), it is important to consider the experimental detail underlying the above conclusions. In effect, transferring 1-2×10^6 peritoneal or splenic B-1 cells (with supporting bone marrow) to irradiated recipients results in the essentially complete and permanent reconstitution of the B-1 population in the peritoneal cavity and apparently at all other sites to which B-1 cells migrate (A. B. Kantor, A. M. Stall, S. Adams, L. A. Herzenberg, in preparation). Conservative estimates indicate that there are roughly 7-10×10^6 B-1 cells in the adult BALB/c mouse and thus that the transferred B-1 cells must have increased their numbers by at least 3-5 fold. Since these estimates take into account the number of B-1 cells in the spleen and the peritoneal and pleural cavities but do not include the number of plasma (or other) cells derived from B-1 cells in sites like the spleen and gut, it is likely that the transferred B-1 population expands considerably more than we estimate.

In contrast, data from transfers of conventional B cells indicate that roughly half the injected B cells are recoverable in the recipient several months after transfer (these do not include plasma cells, etc). For example, Sprent and colleagues report the presence of roughly 3-5×10^6 donor B cells in SCID recipients that received 10^7 lymph node B cells (65). Similarly, we estimate that we recover roughly 1-2×10^6 conventional B cells in BALB/c mice that received either 2×10^6 lymph node B cells or a similar number of FACS-sorted splenic conventional B cells (A. B. Kantor, A. M. Stall, S. Adams, L. A. Herzenberg, in preparation). (Note that better Ig-allotype specific detection is required to identify these cells in reconstituted irradiated recipients than in SCID recipients because of the large number of B cells derived from the obligatory cotransferred bone marrow.) Thus while the B-1 cell population expands 3-5 fold in an adoptive recipient,

conventional B cell populations tend to shrink in size. This difference, while not overwhelming like the expansion of transferred stem cells, is an important distinction between B-1 and conventional B cells, particularly in light of evidence indicating that the B-1 population formed in neonates persists for the life of the animal (see Feedback).

Measurement of B cell turnover by the incorporation of BrdU is consistent with the above results. B-1 cells turnover at about 1% per day, based either on measurements of total PerC B cells (59, 60) or histologically identified B-1 cells (58, 58a). Conventional B cells have a similar turnover rate (59, 60). Measurement of peritoneal B cells in $S+G_2/M$ phases of the cell cycle yields figures consistent with these data (66, 67). Reports suggesting much higher values for peritoneal B cells in $S+G_2/M$ phases of the cell cycle (~20%) (47, 61) may be explained by technical problems, e.g. the failure to exclude doublets from the FACS analysis. Freitas and colleagues, using clever but perhaps riskier systems, also dispute the 1% per day turnover estimate for peripheral B cells (68, 69) as being at least 10-fold too low (68, 69). Further discussion of the many, often conflicting, turnover experiments are beyond the scope of this review.

Feedback Regulation of B-1 Development

The studies discussed above focus on a central question for peripheral B cell dynamics: how important are the processes of self-replenishment and de novo differentiation from progenitors in the "turnover" of B-1 and conventional B cells in intact animals. Answers to this question, unfortunately, are difficult to obtain from cell transfer studies since interpretation of the data requires several key assumptions, e.g. that the "drainage" from the B cell populations into dead cells, plasma cells, etc, is equivalent for conventional B cells and B-1 cells and can therefore be ignored when estimating population expansion.

Results from studies investigating the in situ depletion and recovery of the B cell populations following neonatal treatment with anti-Ig antibodies provide a clear statement on this issue. In essence, Lalor and coworkers in our laboratory have shown that a feedback mechanism that regulates the development of B-1 cells from immature progenitors prevents the emergence of newly differentiated B-1 cells (both B-1a and B-1b) but does not interfere with the development of conventional B cells (44, 70). These findings indicate that B-1 development from undifferentiated progenitors terminates in intact mice somewhere between 3 and 6 weeks of age, whereas conventional B cells continue to develop from immature progenitors throughout the life of the animal.

These studies confirmed earlier findings showing that treatment of neonatal inbred mice with anti-IgM antibodies depletes all B cells, and that normal numbers of B cells return after the treatment Ab disappears (71). In addition, we showed that this recovery extends to both the B-1 and conventional B cells (when all B cells have been depleted), and that monoclonal antibodies to allotypic determinants on IgM (i.e. anti Igh-6b) can also be used to deplete B cells. Thus, the stage was set for comparing the recovery of B-1 and conventional B cells both in *allotype homozygotes*, where the treatment Ab depletes all B cells, and in *allotype heterozygotes*, where the treatment Ab depletes only half of the B cells (i.e. those that express the reactive IgM allotype).

Studies with both kinds of mice yield essentially the same result: conventional B cells recover to normal frequencies shortly after the treatment Ab disappears; B-1 cells, in contrast, only recover when there are no B-1 cells in the animal. B-1 cells fail to recover in allotype heterozygotes, in which only half the B cells (conventional and B-1) are depleted by the treatment Ab. Similarly, they fail to recover in Ab-treated homozygotes in which mature allotype congenic B-1 cells (or a benign B-1 cell tumor) have been introduced during the neonatal period. The presence of mature B-1 cells is necessary and sufficient to prevent the de novo development of B-1 cells in intact animals.

The block in B-1 development proved to be permanent both in the Ab-treated allotype heterozygotes and in the treated homozygotes in which the B-1 cells were restored. More than 6 months after the treatment antibody disappeared and the depleted conventional B cell population recovered, B-1 cells expressing the reactive IgM allotype remained below detectable levels. Thus, we conclude that the B-1 population that develops during the first few weeks of life in normal animals prevents the subsequent entry of newly arisen B-1 cells into the peripheral pool throughout life.

EVIDENCE FOR DISTINCT B CELL PROGENITORS

The dramatic differences between B-1 and conventional B cells discussed above support the idea that they belong to separately developing lineages. The first actual data indicating that this lineage distinction exists, however, came from early cell transfer studies which demonstrated that adult bone marrow readily reconstitutes conventional B cells but only poorly reconstitutes $CD5^+$ B cells (14). Now, some seven years later, a variety of studies confirm and extend this initial hypothesis, demonstrating that conventional B cells and B-1 cells belong to separate developmental lineages (62, 72–75), and suggesting a similar lineage split between B-1a and B-1b cells (62). Rather than discussing these lineage studies in their historical order, we have chosen to organize this section to consider data demonstrating (i) that fetal omentum contains progenitors for B-1 cells

but not conventional B cells, (ii) that fetal liver contains progenitors for both lineages, (iii) that progenitors for B-1 cells, particularly B-1a (CD5$^+$) cells, are depleted in adult bone marrow, and (iv) that FACS-isolated pro-B cells from fetal liver and adult bone marrow are committed to develop, respectively, into B-1 and conventional B cells. It should be noted that we use "progenitors" in a broad sense; the term may include cells ranging in potency and commitment from HSC to pro-B cells. When the data warrants, we employ more specific designations.

Progenitors in Fetal Liver and Omentum

FETAL OMENTUM CONTAINS PROGENITORS FOR B-1 CELLS Solvason and colleagues have shown that 13-day fetal omentum reconstitutes B-1a and B-1b cells *but not conventional B cells* when grafted under the kidney capsule of (or suspended and transferred into) SCID mice (75–78). Since omental tissue at this fetal age does not contain Ig$^+$ cells (77), these findings demonstrate (i) that a distinct site associated with the mesodermal-peritoneal lining houses Ig$^-$ progenitors specifically committed to differentiate to B-1 cells, (ii) that such progenitors exist, and (iii) that these progenitors develop in adoptive hosts according to their original commitment.

The specific progenitors responsible for the B-1 cell reconstitution have not been identified; they could be HSC, lymphoid progenitors, pro-B cells (see Isolation), or a mixture. The fetal omentum also contains progenitors for T cells, demonstrable by cografting fetal omentum with fetal thymus from a genetically distinct donor. This suggests that at least some progenitors in the omentum are not yet committed to the B cell lineage. These findings extend pioneering work by Kubai and Auerbach showing that fetal omentum is a source of lymphocyte progenitors in the mouse (79).

FETAL LIVER CONTAINS PROGENITORS FOR B-1 AND CONVENTIONAL B CELLS Like fetal omentum, fetal liver (13 and 14 day) does not contain Ig$^+$ B cells (80) and readily reconstitutes both B-1a and B-1b cells. However, unlike fetal omentum, fetal liver also reconstitutes conventional B cells (62, 76). Thus, the reconstitution with this tissue comes closest to restoring the normal B cell population frequencies, since transfers of fetal liver reconstitute B-1b and conventional B cells fully and B-1a cells to about half their normal level (62).

Data from the 13 and 14 day fetal liver transfers are consistent with the existence of either one or two B cell progenitors in fetal liver. That is, fetal liver could either contain a single progenitor capable of reconstituting all B cell lineages, or it could contain multiple progenitors committed to develop into distinct B cell lineages. The data from the omentum transfers argue in favor of the latter hypothesis because the 13 day omental tissue,

which is contiguous with the fetal liver capsule, contains only the progenitors for the B-1 lineage(s). In fact, it is possible that the progenitors for B-1 and conventional B cells are actually anatomically separate in the fetal liver, with the progenitors for conventional cells located in the interior of the liver and the progenitors for B-1 cells associated with the capsule. Resolution of this question, however, requires the development of demanding dissection techniques.

PERITONEAL PROGENITORS FOR B-1 CELLS? Marcos et al have presented preliminary data suggesting that there is an adult source of B-1 cells (B-1a and/or B-1b) associated with the peritoneal cavity, perhaps the adult omentum correlated tissue (81). Repeated washing of the peritoneal cavity leads to a loss of B-1 cells. After this in vivo peritoneopheresis is stopped, B cells are reported to return to the peritoneal cavity, first as B220$^+$, IgM$^-$ "pre-B" cells and then as IgM$^+$ B-1 cells. If these results are confirmed, they suggest an adult source of B-1 progenitors that might function in the event of extreme B-1 cell depletion.

Progenitors in Bone Marrow

Since the early work by Hayakawa and Hardy, which demonstrated that bone marrow fully reconstitutes conventional B cells but largely fails to reconstitute B-1 cells (particularly B-1a cells) (14), a variety of bone marrow transfer studies aimed at answering more subtle questions about the nature of the B cell progenitors in bone marrow have been completed (34, 62, 63). Two new conclusions can be drawn from this work. First, although the new data show that there is more variation in the low levels of B-1a reconstitution from bone marrow than previously recognized, these findings still clearly confirm the earlier evidence indicating that bone marrow contains very little progenitor activity for B-1a cells. Second, the new data confirm and extend earlier evidence (44) indicating that substantial progenitor activity for B-1b cells is present in adult bone marrow and functions when there are very few B-1a cells in the animal (62).

BONE MARROW CONTAINS PROGENITORS THAT FULLY RECONSTITUTE CONVENTIONAL B CELLS The total number of splenic T cells and conventional B cells routinely returns to normal levels or above in bone marrow recipients. However, while conventional B cells comprise 10–20% of the lymphocytes in normal Balb/c PerC, they represent 50–60% of the PerC lymphocytes in bone marrow recipients (62). This increased frequency of conventional B cells mainly reflects the failure to reconstitute normal numbers of B-1a cells.

BONE MARROW CONTAINS VERY FEW PROGENITORS FOR B-1a CELLS In our hands, the level of peritoneal B-1a ($CD5^+$) cells recovered from adult bone marrow transfers is roughly 5% of the number of B-1a cells in normal (intact) animals. This low level B-1a cell reconstitution could be due wholly or in part to rare (self-replenishing) B-1a cells located in the bone marrow; however, since transfers of $B220^-$ bone marrow cells also result in similar low level B-1a reconstitution (58, 59), it is likely that a low frequency of B-1a progenitors survives into adulthood and is revealed in adoptive recipients.

In different experiments involving both BALB/c and CBA mice tested from 2–8 months after transfer, the number of B-1a cells recovered ranges from <2% to 15% of normal B-1a levels (62, 63) (A. B. Kantor, A. M. Stall, S. Adams, L. A. Herzenberg, in preparation). The high levels of B-1a cells recovered could be accounted for by the occasional presence of B-1a clonal neoplasms in the bone marrow source, because these clones tend to expand extensively in adoptive recipients (82). In addition, these high levels could be due to exceptionally high levels of circulating B-1a cells or to B-1a cells localizing in the bone marrow. However, as indicated above, it is likely that the variation in the number of B-1a cells recovered in bone marrow recipients is largely determined by the number of B-1a progenitors that persist in adults.

Other groups claim to obtain substantially higher B-1a reconstitution from bone marrow. Unfortunately, some of these studies have serious technical flaws (83, 84), and others are too incomplete to fully evaluate (85–87). For example, in striking contrast to other published data (14, 34, 44, 62, 63, 72, 73), one study concludes that bone marrow reconstitutes B-1a cells as well as PerC in irradiated recipients (83). The FACS data in this study, however, were *analyzed inappropriately*: a and b allotype B cells were not adequately resolved; gates were chosen incorrectly, perhaps in part because of the limitation of the contour program used; and the panel of staining reagents was too limited. Another study, which reports moderate bone marrow reconstitution of B-1a cells (84), has similar technical difficulties. This study reports approximately equal levels of IgM^+, $CD5^+$ cells following bone marrow transfers in both the spleen and peritoneum of the *irradiated recipients. This* contradicts the well-established finding that B-1a cells localize to the peritoneum after transfer (14, 34, 44, 62, 63, 67, 72, 73).

When examined closely, none of the putative findings in the above studies seriously challenges the argument that bone marrow is a poor source of progenitors for reconstituting B-1a cells. In contrast, well-grounded data repeatedly demonstrate that bone marrow largely fails to reconstitute B-1a cells (14, 34, 44, 62, 63, 72, 73). Since these studies show that conventional B cells are fully reconstituted in the same bone marrow recipients in which B-1a reconstitution largely fails, we interpret this evidence as indicating that conventional B cells derive from different progenitors than B-1a cells.

BONE MARROW TRANSFERS DISTINGUISH PROGENITORS FOR B-1a AND B-1b CELLS We repeated our earlier transfer studies and more closely defined the kinds and frequencies of B cells reconstituted from adult bone marrow. These studies suggested the division of the B-1 population into two B-1 lineages, now provisionally called B-1a and B-1b.

The first evidence suggesting distinct developmental differences between B-1a and B-1b cells came from feedback regulation studies showing that the B-1 population that recovers following neonatal B cell depletion by anti-IgM antibody treatment (of allotype homozygotes) consists largely of B-1b cells. This evidence suggested that functional progenitors for B-1b cells persist longer into adulthood than progenitors for B-1a cells. These studies also established B-1b cells as a distinct population by showing that FACS-sorted B-1b cells are fully capable of self-replenishment in adoptive recipients, and neither derive from nor give rise to B-1a cells (44, 70).

Data from our recent bone marrow transfer studies confirm the independent progenitor origins of B-1a and B-1b cells (34, 55, 63). In agreement with previous data, these studies show that B-1a cells are very poorly reconstituted by progenitors from this source. In addition, however, they show that B-1b cells are routinely reconstituted in bone marrow recipients and, on average, reach half their normal frequency (40, 58, 84, 30, 59). This does not amount to a large reconstitution of the overall B-1 population, because B-1b cells usually represent less than a quarter of this population in the Balb/c animals used for this study. These bone marrow-derived B-1b cells also replenish themselves when peritoneal cells are transferred into a second set of recipients. (A. B. Kantor, A. M. Stall, S. Adams, L. A. Herzenberg, in preparation).

The *reconstitution of B-1b cells* was difficult to detect in the earlier studies, largely because these studies used a more limited set of cell surface markers and a more limited FACS instrument (two- rather than three-color) to characterize the B cell populations in transfer recipients. In contrast, current FACS and reagent technology reveals the reconstitution of B-1b cells quite clearly and leaves little doubt that they are reconstituted much more efficiently (per cell transferred) by adult bone marrow than are B-1a cells.

This reconstitution data is consistent with the idea that cells expressing

the B-1b-phenotype are derived from two B cell progenitors—one that is most active early in ontogeny and also produces B-1a cells, and one that is active later in ontogeny and also produces conventional B cells. However, evidence from the feedback inhibition studies makes this dual-progenitor hypothesis less attractive. These studies, which show that the development of all B-1b cells is sensitive to feedback inhibition by mature B-1 cells, make it unlikely that a proportion of B-1b cells are derived from the same progenitors that give rise to conventional B cells. Therefore, we interpret the data from the bone marrow transfer studies, which suggest that functional progenitors for B-1b cells survive longer into adulthood than progenitors for B-1a cells, as indicating that B-1a and B-1b cells are derived from independent progenitors and hence that these cells belong to distinct developmental lineages (Figure 2).

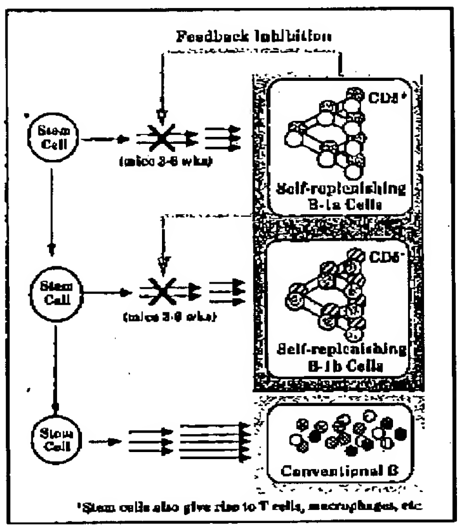

Figure 2 Development and feedback regulation of B cell lineages. The progenitor studies reviewed here demonstrate that B-1a and conventional B cells are distinct lineages. Bone marrow transfers also suggest that B-1b cells constitute a distinct lineage. Feedback inhibition regulates the *de novo* production of both B-1a and B-1b cells.

Cotransfer of Progenitors in Fetal Liver and Adult Bone Marrow

Since B-1a cells readily develop from fetal liver in adoptive transfers, thei limited reconstitution from adult bone marrow is not due to condition inherent in the recipient environment per se. However, the environmen in a recipient being reconstituted with fetal liver is not necessarily the sam as the environment in a recipient being reconstituted with bone marrow Fetal-liver derived cells or cell products could be required to support th development of B-1a cells; or, bone-marrow derived cells or cell product could block B-1a cell development. Therefore, before we finally conclude that progenitor activity for B-1a cells is deficient in adult bone marro (62), we co-transferred fetal liver and adult bone marrow and demon strated that B-1a progenitors in the co-transferred recipient develop nor mally and exclusively from the fetal liver source. These studies ar described in the next section.

B-1a PROGENITORS ARE DEPLETED IN ADULT BONE MARROW In the studie referred to above, we cotransferred 14-day fetal liver (BAB, Igh b-allotype and adult bone marrow (Balb/c, a-allotype) into irradiated recipients (62) Analysis of the recipients demonstrated that bone marrow does not contai cells that limit the development of B-1 cells from their progenitors (an hence hide the fetal liver progenitors); and fetal liver does not contain cell that enhance the development of B-1a cells (and therefore reveal crypti progenitors for these cells. In essence, data from these studies showed (i that fetal liver and bone marrow reconstitute the same proportion o peritoneal B-1a, B-1b, and conventional B cells whether transferred to gether or separately; (ii) that bone marrow reconstitutes the B-1b cell better than B-1a cells, as in the separate transfers described above; an (iii) that the ratio of bone marrow-derived B-1b cells to B-1a cells i cotransfer recipients is equal to the ratio observed when bone marrow i transferred alone. Thus, we conclude that bone marrow is clearly deficien for progenitors for B-1a cells.

B-1 AND CONVENTIONAL B CELL PROGENITORS MAY BE DISTINCT IN FETAL LIVE The cotransfer studies discussed above incidentally provided evidenc demonstrating that fetal liver transfers can reconstitute B-1 cells withou reconstituting conventional B cells. That is, in some cotransfer recipient (3/13), fetal liver readily reconstituted B-1 cells but failed to reconstitut conventional B cells. All conventional B cells in these recipients wer derived from the cotransferred bone marrow (62). The simplest explan ation for these findings is that the progenitors for B-1 cells and conventional B cells are distinct in fetal liver; and that the progenitors for B-

1 cells were abundant in the fetal liver suspension that was transferred, whereas the progenitors for conventional B cells were rare enough to fail to be expressed in some recipients.

Isolation of B Cell Progenitors

The question of separate progenitors, and hence separate lineages, is best addressed by contrasting the developmental potential of the earliest fetal and adult progenitors in the B cell developmental pathway, i.e. hematopoietic stem cells (HSC) and their committed offspring, particularly pro-B cells. Hardy and Hayakawa have made substantial progress in this area (72–74, 88).

First, Hardy and Hayakawa demonstrated that HSC population(s) from either neonatal liver or adult bone marrow readily reconstitute conventional B cells (in irradiated SCID recipients), whereas B-1 cells are only reconstituted by HSC isolated from neonatal liver. Second, they showed that FACS-sorted pro-B cells from adult bone marrow mainly give rise to conventional B cells whereas pro-B cells from neonatal sources give rise to B-1 cells. This series of progenitor studies, which definitively establishes the independent lineage origins of B-1a and conventional B cells, is summarized in the sections that follow.

RECONSTITUTION WITH FACS-SORTED HSC As expected, when adult bone marrow HSC are enriched by FACS-sorting Thy-1^{lo}/Lin$^-$ (B220$^-$, CD4$^-$, CD8$^-$, etc) cells and transferred to SCID recipients, they reconstitute B cell populations similar to unsorted and B220$^-$ adult bone marrow (compare 62, Figure 1, and 72, Figure 1). Notably, conventional B cells are fully reconstituted, B-1b cells are reconstituted at substantial frequencies, and a small but detectable number of B-1a cells are also derived from the donor source. Thus, differentiation from HSC is sufficient to account for the limited reconstitution of B-1 populations from adult bone marrow discussed above.

These transfers also provide evidence for distinct B-1 progenitors in the HSC fraction sorted from neonatal liver. FACS-sorted fetal liver HSC populations contain progenitors for all B cell populations. However, while transfers of 50,000 Thy-1^{lo}/Lin$^-$ cells reconstitute both B-1 and conventional B cells, transfers of small numbers (500) of the FACS-sorted HSC only reconstitute conventional B cells (72, 73). These data are consistent with the selective reconstitution of B-1 cells that we observed in several recipients of fetal liver (cotransferred with adult bone marrow). Taken together, these findings add weight to the idea that progenitors for B-1 cells are distinct from progenitors for conventional B cells in fetal liver.

RECONSTITUTION STUDIES WITH FACS-SORTED PRO-B CELLS Hardy and Hayakawa also demonstrated that the difference in B lineage commitment observed for HSC in adult bone marrow and fetal liver is reflected by the commitment of differentiated pro-B cell from these tissues (72–74, 88). They first used multiparameter FACS analysis and sorting to define a differentiation pathway for B220$^+$ pro-B and pre-B cells (88) based on the differential cell surface expression of BP-1 (89), CD43 (leukosialin, S7—37, 90), and heat stable antigen (HSA, 30F1). They defined the differentiation status of the isolated populations with respect to Ig rearrangement: pro-B cells have D_H-J_H but not V_H-D_HJ_H rearrangements, whereas pre-B cells show the full $V_HD_HJ_H$ rearrangements.

Most significantly, sorted adult bone marrow pro-B cells (B220$^+$, CD43$^+$, HSA$^+$) in the above studies reconstitute mostly conventional B cells, while similarly sorted fetal liver pro-B cells yield only B-1 cells when transferred into lightly irradiated SCID recipients. Thus, pro-B cells are committed to particular lineages when isolated from fetal versus adult sources. The repopulation of conventional B cells from adult bone marrow pro-B cells peaked around 2–3 weeks after transfer and subsequently decreased. The B-1 cells repopulated from fetal liver pro-B cells peaked by 2 months and remained constant thereafter. The repopulation kinetics from fetal and adult pro-B cells are consistent with and provide further evidence of the self-replenishing capabilities of B-1 cells.

The distinct differentiation potential of fetal liver and adult bone marrow pro-B cells was also demonstrated with short-term stromal layer cultures (74). Fetal liver pro-B cells yield mostly CD5$^+$ B cells whereas adult bone marrow pro B cells yield mostly CD5$^-$ B cells. Thus, in vitro results are completely compatible with the results from in vivo studies. The development of the CD5$^+$ (B-1) B cell population in the fetal pro-B cells cultures bears on another question of importance. It demonstrates that the phenotype of B-1 cells is not defined by in vivo influences, e.g. interaction with maternal antibodies or with self-antigens other than those expressed by the restricted set of cells in the stromal culture.

B CELL ANTIBODY REPERTOIRE

Developmental differences may be important in determining functional distinctions among the B cell lineages. Here we review some of the evidence for differences between the repertoires of B-1 and conventional B cells with respect to isotype, specificity, response and rearrangement machinery. We also consider the influence of selection on the expressed repertoire of the individual lineages and possible influences of selection on the phenotype of B cells.

This discussion is necessarily incomplete because current information is not sufficient to draw conclusions on key issues relevant to our focus here, that is, the lineage origins of B cells. For example, although B-1 cells have been clearly shown to predominate in the response to certain antigens, further studies are required to determine whether these functional differences reflect differences in the potential of the lineages to express the particular Ig rearrangements used in these responses. Similarly, although initial studies suggested that V gene representation in the peripheral B-1 cell repertoire is considerably more restricted than in the conventional B cell repertoire, subsequent studies reopen this question by demonstrating substantially more diversity in the B-1 cell repertoire, with respect both to V_H gene representation and to N-region insertion.

Resolution of these issues has been hampered by the lack of adequate methodology to define the native repertoires of the B cell lineages. Much of the early V gene data comes from hybridomas or mitogen-stimulated B cells, which of necessity define selected repertoires dependent on functional response potential. Other data, based on cDNA amplification of sorted or ontologically isolated B cell populations, is skewed toward the most abundant messages in a particular population. Overall, therefore, although considerable data has been amassed, the native (and locally selected) repertoires of the lineages have yet to be clearly defined.

The question of B-1 and conventional B cell participation in T-dependent and T-independent responses also has yet to be fully resolved. B-1 cells are responsible for producing many commonly studied autoantibodies and antibacterial antibodies; however, the assumed extension of this evidence to the idea that B-1 cells only produce T-independent responses is incorrect. Thus, although there are a number of generally accepted ideas about the repertoires of B-1 and conventional B cells, corrections and caveats apply to many of the interpretations given to the data. These and related issues are discussed in the sections that follow.

The B-1 Antibody Responses

IMMUNOGLOBULIN ISOTYPES Although the antibody responses in which B-1 cells have been studied tend mainly to be IgM (e.g. to bromelain treated erythrocytes), B-1 cells can produce all Ig isotypes. They make major contributions to serum IgM, IgG_3, and IgA (33, 76, 91) and produce a large percentage of the IgA-producing plasma cells in the gut (54–56, 92). The B-1 cell contribution to total serum IgM is dramatically demonstrated in mice treated with anti-IL-10 antibody (92a). B-1 cells, which are the main source of B cell-derived IL-10 (92b), are completely depleted from the peritoneum by the anti-IL-10 treatment and serum IgM is drastically reduced, to < 10% of normal. Conventional B cells, which remain in the treated mice, are still able to make specific IgM in response to TNP-KLH.

Studies with B-1 cell lines indicate that cytokines (e.g. IL-4) regulate the switching of stimulated B-1 cells to the more advanced isotypes (93–95). Since T cells are likely to be the major source of such cytokines in antibody responses, these isotype switch data suggest that the characteristics of B-1 antibody responses are regulated by T cells in much the same manner as the responses of conventional B cells are thought to be. However, the question of affinity maturation and somatic mutation in the B-1 cell-derived IgG and IgA-secreting plasma cells is still unresolved.

CLONAL POPULATIONS OF B-1 CELLS Virtually all mice over the age of 15 months have clonal populations of B-1 cells detectable in Southern gel analyses of splenic or peritoneal lymphocytes (96). These clonal B-1 populations, which are also detectable by FACS analysis when they become large, are present in many older mice (> 5 months of age) and can even be found in neonates from some mouse strains (e.g. NZB) (82). They appear quite frequently in irradiated recipients reconstituted with peritoneal B cells from older mice (82) and in nonirradiated neonatal mice injected with peritoneal cells (67). On occasion, spleen and bone marrow can also yield clones (82) (and unpublished observations). Unfortunately, when present, these clonal populations skew the results of repertoire analyses and can lead to erroneous views of the overall B cell repertoire. V gene studies have associated B-1 populations with the expression of a limited, germline repertoire; however, the presence of B-1 clonal populations may have overemphasized the extent of this restriction in some studies (82, 96–99). The repertoire in unmanipulated young mice appears to be more diverse, at least within the J558 family (100).

The influence of feedback regulation and the emergence of clonal populations on the development of the B-1 repertoire are summarized in Table 2. In essence, the B-1 repertoire is fixed early in development and becomes progressively restricted as animals age, because new entrants to the B-1 pool are prevented (due to the feedback mechanism), and clonal populations expand to occupy a progressively greater proportion of the pool.

B-1 ANTIBODY RESPONSES B-1 cells respond well to some multivalent antigens (T independent), especially in connection with the production of auto- and anti-bacterial specificities. They produce the major response to microorganismal coat antigens such as lipopolysaccharide (101) α1-3 dextran (67), phosphorylcholine (PC, T15, idiotypes; 101a) and undefined determinants on *E. coli* (102) and Salmonella (103). In addition, they respond to another bacterial coat component, phosphatidylcholine (PtC), which is often measured as reactivity to bromelain-treated mouse red

Table 2 Repertoire development in B cell lineages

Stage	Age	B-1 cells	Conventional B cells
Fetal	12–15 days	Progenitors (HSC) appear in the liver and omentum	Progenitors appear in the liver (not in omentum)
	> 16 days	Progenitors (including pro-B cells) begin to give rise to B-1 cells	
		Selective forces start to shape the repertoire potential	
Postnatal	0–4 weeks	Progenitors continue to give rise to self-replenishing B-1 cells; population approaches adult size	Population starts to enlarge
Adolescent	4–8 weeks	Feedback inhibition blocks new development from progenitors	Population approaches adult levels; de novo differentiation from progenitors continues
		Repertoire potential becomes fixed	
Adult	8–20 weeks	Individual clones expand or are deleted	Population reaches maximal levels (12–14 weeks); de novo differentiation from progenitors continues
		Repertoire becomes progressively more restricted	
Elderly	> 20 weeks	Hyperplastic and neoplastic (B-CLL) clones appear	

blood cells (104, 105) or PtC-containing liposomes (106). This reactivity uses V_H11 and V_H12 almost exclusively (107–111a) and accounts for ~10% of the peritoneal B-1 cells. B-1 cells also produce other autoantibodies, e.g. to thymocytes (112).

T dependent vs T-independent responses The ready responsiveness of B-1 cells to bacterial coat antigens and other typical T-independent (TI) antigens appears to have led to the erroneous idea that responsiveness to T-dependent (TD) and TI antigens distinguishes B-1 cells from conventional cells. It is true that B-1 cells do not respond very well to certain laboratory antigens commonly used to study TD antibody responses, e.g. B-1 cells respond poorly to sheep erythrocytes and TNP (22, 105) and NP (67) haptens in TD (protein coupled) form. However, B-1 cells also do not produce a clearly detectable plaquing response to TNP or NP in a TI (Ficoll coupled) form (21, 67, 105). Thus the ability to respond does not hinge simply on the form in which an antigen is presented.

The lack of response to the TNP hapten could reflect the state of the B-1 repertoire and/or an inability to stimulate somatic mutation and affinity maturation in B-1 cells with either the TI or TD form of TNP. In vitro LPS stimulation studies reveal a high frequency of FACS-sorted B-1 cells that produce antibodies that bind to TNP; however, these antibodies are broadly reactive and have a low affinity. Thus, they differ from the relatively high affinity, fine specificity antibodies that are elicited even in a primary TD anti-TNP response produced in vivo by conventional B cells (113).

B-1a cells do, however, produce TD responses to certain antigens. For example, they are the major source of the dominant $T15^+$ idiotype in the antibody response to phosphoryl choline (PC) (101a, 114), which Taki et al have demonstrated is stimulated by the TD antigen PC-KLH (115). Also, A/WySNJ mice, which have B-1 cells but are deficient in conventional B cell development, make good primary IgM responses to both TD and TI antigens, but poor secondary IgG responses (116, 117). Thus, although they are selective with respect to antigen, B-1 cells are capable of making both TD and TI responses.

T cells clearly influence other aspects of antibody production by B-1 cells. Hnetz and coworkers described the dependence of the LPS-driven anti-PtC response on CD4 T cells (117a). Taki et al transferred FACS-sorted B-1a cells into SCID mice either alone or with T cells. The T cells enhanced Ig production by B-1a cells and induced switching from IgM to other isotypes, including IgG_1 (115). Similar results were observed in omentum-thymus corecipients in that the addition of the thymic tissue to the graft resulted in substantially increased production of IgG isotypes (76).

Immunoglobulin Rearrangement

Is the machinery that controls Ig heavy and light chain rearrangement different for B-1 and conventional B cells? Both the RAG-1 and RAG-2 gene products are required for successful Ig rearrangement in any type of B cell (118, 119), and no differences have, as yet, been reported for these enzymes in B-1 and conventional B cells. In contrast, terminal deoxynucleotide transferase (TdT), which inserts noncoded nucleotides (N-regions) at the gene segment junctions during rearrangement (120, 121), appears to be absent in the progenitors of the B cells that develop early in fetal life (122, 123). Thus, questions have arisen as to whether all committed progenitors of B-1 cells selectively lack TdT activity and hence whether the absence of Ig N-region insertions is a defining characteristic for this lineage (see Origins, below).

N-REGION INSERTIONS Several groups have demonstrated that fetal and neonatal V_H-D and D-J_H junctions have very few N region insertions whereas most such junctions recovered from adults have longer N regions (124–128). As a consequence of this absence of N-region insertions early in ontogeny, rearrangement of certain V_H-D-J_H gene segments are favored, i.e. those with short sequence homologies (127, 129). Together, these rearrangement mechanisms potentially restrict the early B cell repertoire and thus may have a disproportionate effect on the Ig produced by B-1 cells.

The work published by Rajewsky's group is most informative with respect to N-region insertions because they use PCR amplification to construct cDNA libraries of expressed genes from FACS-sorted B cell subsets (127, 130). Data from these studies show that N-region sequences are rarely inserted at the V_H-D and D-J_H junctions of B-1a cells present in the spleen at four days after birth (average N = 0.6 at the V_H-D and 0.0 at the D-J_H junctions). Peritoneal B-1a cells present at one month of age, in contrast, have more N-region insertions (2.2 at V-D and 0.7 D-J_H) and are intermediate in this sense between the neonatal B-1a cells and conventional B cells isolated from spleen either at one (4.6 V_H-D and 2.8 at D-J_H) or at four months (4.7 at V_H-D and 2.4 at D-J_H). Gu et al also analyzed sequence data from the CH series of B-1 cell lymphomas (97) and showed that many of these neoplasms, which are similar to human B-CLL, lack N-region insertions and hence appear to have arisen early in ontogeny.

For the evaluation of self-replenishing B-1a cells from adults, Gu et al rely on sequence data from hybridomas prepared following LPS stimulation of spleen and PerC of 8-month-old allotype chimeras which, as neonates, were injected with peritoneal cells from 6-10 month old allotype

congenic donors (99). The average length and distribution of the N-region insertions in these hybridomas is similar to N-region size in adult conventional B cells, suggesting that many B-1 cells develop from TdT-expressing B-cell progenitors, which probably begin to function near birth.

The findings reported by Gu et al do not necessarily reflect the size and distribution of N-regions in B-1a cells in normal adult animals, because the highly manipulated B-1 populations in these chimeric mice are likely to be biased. Therefore, we believe it is likely that further analysis will demonstrate that adult B-1 populations, like the FACS-sorted B-1 cells analyzed from 1-month-old animals, have on average more N-region insertions than fetal B-1 populations but fewer such insertions than conventional B cells. Some of the B-1a cells present in the adult may lack N-regions and may have survived via self-replenishment since birth.

Contrasting the representation of N-region insertions in the various B cell lineages in adults may be further complicated by selective processes. A comparison of functional and nonfunctional rearrangements in the 7183 V_H family shows significantly more N-region diversity in rearrangements on the nonfunctional chromosome than on the functional chromosome, in both fetal and adult splenic B cells. Since the analysis of adult splenic B cells most likely is weighted in favor of sequences from conventional B cells, these data suggest (i) that rearrangements associated with a lack of N-region sequences are not restricted to the fetal period; (ii) that rearrangements in both B-1 and conventional B cells may lack N-region sequences; and (iii) that selective forces tend to favor B cells expressing Ig with little or no N-region insertion (131).

J_H PROXIMAL V_H FAMILIES There is considerable evidence demonstrating that the V_H repertoire in fetal and neonatal B and pre-B cell populations is biased towards J_H proximal families while the V_H repertoire in adult splenic B cells is more randomized (normalized) with a heavy expression of genes from the distal (J558) family (132–138). A bias in fetal and neonatal B cells could be related to factors influencing the development of the B-1 cell repertoire, since B-1 cells tend to predominate early in ontogeny and have a functionally restricted repertoire with a high level of self-reactivities. However, mRNA analysis of LPS stimulated conventional and B-1a cells from adults demonstrates that B-1 cells use the whole spectrum of V_H families, without preference for J_H proximal ones (138a,b). The high frequency of V_H11, V_H12 (anti-PtC) and 3609 (anti-thymocytes) gene usage suggests a lack of preference for J_H proximal families by B-1 cells. Short sequence homologies bias junctional recombination of extra-chromosomal substrates most readily in cell lines low in TdT expression

(138c). This mechanism may actually be more important in biasing the early Ig repertoire than is chromosome position (129). Homology-directed recombination is likely to be important in generating some B-1 cell specificities such as anti-PC (T15 idiotype).

Recent evidence indicates that the bias for J proximal V_H families also occurs in developing B cells in adult bone marrow (132, 136). The demonstration that the position-dependent V_H family bias occurs in newly arising B cells at all stages of ontogeny argues strongly that the relatively increased frequency of distal V_H genes in the spleen must reflect the operation of selective (or other) mechanisms that control the entrance or the retention of B cells in the spleen and at other sites.

Selection of Peripheral B-1 and Conventional B Cells

There is no doubt that selection is important in determining the repertoire of both B-1 and conventional B cells. For example, by comparing the V_H repertoire of pre-B and mature B cells from sorted bulk populations, Rajewsky's group has shown that pre-B cells from neonatal liver or adult bone marrow utilize a wide range of V_H genes within the large J558 family whereas the set of V_H genes expressed by peripheral B cells, both B-1a and conventional B, is considerably more restricted (100). Thus, the entry of all B cells into the long-lived peripheral B cell pool either requires positive recruitment or occurs after a negative selection phase.

The selection process begins early in development for both B-1 and conventional B cells, at the stage when pseudo light chain (ψL = $\lambda 5$+Vpreß) is expressed on immature B cells in conjunction with μ or D_μ proteins (reviewed in 23). The filling of both the B-1 and conventional B cell compartments is impaired in mice made deficient for $\lambda 5$, but not eliminated (139a). The B-1 cell population reaches full size in the $\lambda 5$ knockout mice more slowly than in normal mice, and the conventional B cell population is still reduced five-fold even at 4 months of age. The B-1 cells might simply accumulate better than conventional B cells because of their greater self-replenishing capabilities, although it is possible that B-1 cells are better able to employ alternative differentiation pathways, which are not dependent on $\lambda 5$ protein.

Since B-1 cells develop early in ontogeny, B-1 repertoire differences may at least in part reflect selection by different endogenous antigens and/or immunoglobulins present in the fetus (78, 139). Such selection appears to play a key role in the recruitment of the fetal B cells that produce the germline-encoded antibody specificities prevalent in the B-1 cell population (in neonates and adults). For example, independently rearranged V_H11 and V_H12 genes are expressed in a large series of anti-PtC (an anti-self specificity) hybridomas and lymphomas (98, 109). PCR

amplification of sorted pre-B and B cells from adult bone marrow indicat that functional rearrangements of this V_H11 also occur frequently in con ventional B cells; however, these B cells are not found in spleen and the do not appear to be selected into the peripheral conventional B cell poo (110).

Kearney and coworkers have shown that neonatal treatment with ant idiotype MAb can deplete specificities and permanently alter the repertoir as measured by idiotype representation (78). Treatment timing is cruci and related to the normal development of antigen-specific precursors (140 The high interconnectivity (idiotype/anti-idiotype) observed in the early cell repertoire appears to play a role in these processes, suggesting th production of these kinds of antibodies may be important in establishin the B-1 repertoire expressed in neonatal animals and adults (140-143 Similarly, maternal antibodies transmitted through the placenta and maternal milk may also influence the characteristics of the B-1 repertoir These kinds of alterations of the neonatal B-1 repertoire may be pa ticularly important, because they effectively perpetuate neonatal immun logic experience throughout life.

B CELL SELECTION IN TRANSGENIC MICE There are a large number of t transgenic mouse strains currently under study in a variety of laboratori (144-151). Many of these strains show B cell developmental defects th alter the relative frequencies of B-1 and conventional B cells and sometim block rearrangement of endogenous Ig. B-1 cells in several strains hav been shown to coexpress endogenous and transgenic Ig or to expre mostly endogenous Ig while conventional B cells in the same anima express only the transgenic Ig (56, 152-154). These aberrations in production undoubtedly reflect the selectability of the transgenic Ig an endogenous Ig molecules expressed by individual B cells. Furthermor they are influenced by the self-replenishing capability of the B-1 cells th are selected into the peripheral pool. However, the operation of the factors does not preclude other differential effects of the transgene, e. selective interference with Ig rearrangement in the development of con ventional B vs B-1 cells.

THE ORIGINS OF B CELL LINEAGES

Prior to the demonstration that progenitors for B-1 cells are distinct fro progenitors for conventional B cells (see Progenitors), there was still roo for a "selection-only" hypotheses that viewed B-1 cells as a type of antige stimulated conventional B cell, "activated" early in ontogeny and selecte to persist via self-replenishment throughout life. This view gained intere

when Wortis and colleagues showed that CD5 expression and other aspects of the B–1a phenotype can be induced by stimulating conventional B cells with anti-IgM antibodies in the presence of certain cytokines (39). However, even this group now agrees that the progenitor studies rule out a simplistic, one-lineage hypothesis (85, 155).

Their current hypothesis (85, 155) proposes two B cell lineages: a fetal lineage, whose TdT-progenitors produce B cells which lack N-region insertions in their rearranged V genes; and an adult lineage, whose TdT$^+$ progenitors produce B cells which contain N-region insertions in their rearranged V genes. Based on the data from in vitro anti-IgM stimulations, they argue that stimulation of B cells of either lineage with multivalent (cross-linking) TI–2 antigens in the presence of cytokines leads to the expression of CD5 and a shift to the entire B–1a cell phenotype. They then argue that fetal lineage B cells are more likely to be stimulated in this way because the Ig molecules they express, which lack N-region insertions, will be strictly encoded by germline genes evolved to recognize TI–2 antigens such as micro-organismal coat molecules and related self-antigens. Thus, the Wortis group proposes that the B–1a population is largely generated early in ontogeny and persists thereafter by self-replenishment, perhaps stimulated by the self-antigens that initially selected them into the B-1 pool; however, newly differentiated B cells from the adult lineage will enter the B-1 population whenever appropriately stimulated.

We view this "TI-2" model of B cell development as possible but not probable. First, current evidence indicates that B-1 and conventional B cell antibody responses do not segregate with respect to sensitivity to stimulation with TI-2 or any other known classification of antigens (see Responses). Secondly, evidence has yet to be presented demonstrating that the stimulation of conventional B cells that induces expression of the B-1a-like phenotype actually generates functional B-1a cells capable of survival and/or self-replenishment in vivo. In fact, Hayakawa et al have shown that essentially all anti-TNP PFC in the spleen are CD5$^-$ following immunization with the classic TI-2 antigen TNP-Ficoll (105) (see Activation). Third, although more work is required to characterize definitively the frequency of N-region insertion sequences in B-1 vs conventional B cells, current data indicates that a substantially higher representation of N-region sequences in Ig produced by B-1 cells is found in animals over 4 weeks of age (see N-Region) than would be predicted from the observed frequency of new entrants into the B-1 population.

Next, data from the feedback regulation studies demonstrate that the entry of new B cells into the B-1a population in intact adult animals is completely blocked. And finally, although some B-1a cells (<10% of the population) appear in bone marrow recipients, their failure to accumulate

over time is inconsistent with the idea that they derive from the adult conventional B cell progenitors, which continuously generate vast numbers of newly rearranged B cells, including some which even have no N-region insertions. Thus current evidence continues to strongly favor the idea that B–1a cells are derived from committed progenitors that do not give rise to conventional B cells; and similarly, that conventional B cells are derived from committed progenitors that do not give rise to B-1a cells.

The Layered Immune System

Current data identify three B cell lineages that appear sequentially, with some overlap, during development. B–1a cells appear sometime after day 16 of fetal life and are readily reconstituted from progenitors in fetal omentum and in fetal and neonatal liver. B–1b cells appear about the same time as B–1a cells (or shortly thereafter). They are readily reconstituted from the fetal and neonatal sources that reconstitute B–1a cells but can also be reconstituted well from progenitors in adult bone marrow. Both B-1a and B-1b cells persist as self-replenishing populations throughout adult life; new entrants into the adult peripheral pool are prevented by a feedback mechanism triggered by the presence of a mature B-1 population. Conventional B cells, in contrast, begin to appear during the post-natal period, are readily replenished in situ from undifferentiated progenitors, and are reconstituted in transfer studies from progenitors present in both fetal and adult sources.

The recognition of distinct B-cell lineages could be strictly interpreted within the framework of B cell development; however, the progenitor studies with FACS-isolated HSC populations from fetal and adult sources suggest a broader context for consideration of these findings (Figure 3). These populations contain pluripotent stem cells that, by definition, also give rise to T cells, erythrocytes, and myeloid cells. Thus the demonstration that HSC from fetal and adult sources give rise to distinct B cell lineages suggests the existence of similar lineages of other kinds of differentiated hematopoietic cells. Since certain of these lineages have already been identified (7, 18), these considerations lead us to propose that evolution has created a layered immune system by successively adding developmental lineages that provide progressively more complex functions (44, 156).

The parallel developmental patterns and repertoires exhibited by T- and B-cell populations/lineages suggest that B–1a cells and early $\gamma\delta$ ($V_\gamma 3$) T cells represent the most primitive "layer" of this immune system. Subsequent layers then might link B–1b cells with of $V_\gamma 4$ cells (157, 158) and, finally, conventional B cells with the remainder of the T cell populations. Data supporting this concept have been reviewed elsewhere (62, 156, 158). For example, *αβ* T cells, like conventional B cells, appear around birth

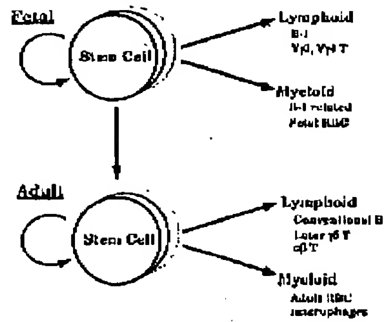

Figure 3 The layered immune system. Transfer studies demonstrate that the stem cells present in fetal life give rise to different sets of hematolymphoid cells than those stem cells present in the adult. The distinct progenitors have been demonstrated for murine B cells (14, 72, 73) and T cells (7, 18), and sheep erythrocytes (10–13)

and become predominant as the animal matures. Both $\alpha\beta$ T cells and conventional B cells, which circulate throughout the animal and predominate in secondary lymphoid organs, can be replenished throughout life by de novo differentiation from stem cells in the bone marrow.

Functional considerations suggest that B-1 cells and early $\gamma\delta$ T cells, by nature of their repertoire and anatomical location, may create a first line of defense against invading pathogens. B-1 cells produce a more restricted set of low-affinity, broad-specificity germline antibodies that react with ubiquitous microorganisms, whereas conventional B cells produce a large, more diverse set of antibodies capable of specific high affinity interactions with particular pathogens. Similarly, the repertoire of the early $\gamma\delta$ T cells is considerably more restricted than the diverse repertoire of $\alpha\beta$ T cells. Thus, the functional distinctions among layers in the immune system are visible both phylogenetically and ontogenically.

In sum, the evolution of the immune system appears to have brought into existence a series of stem cells that sequentially give rise to lymphocytes that are similar to their predecessors but may have added (or lost) functional capabilities. Because the evolutionary success of the latest layer depends on its ability to introduce a selective advantage, each new layer can be expected to increase the sophistication of the immune system with respect to its ability to efficiently protect the animal against challenges within its environment. This concept of an evolutionarily layered immune system presents a framework that unifies data from T and B lineage studies and offers a model that can guide future work.

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TRANSCRIPTIONAL REGULATION OF T CELL RECEPTOR GENES

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Abstract

The diverse lineages of the mammalian hematopoietic system including both B and T lymphocytes are derived from a single mesodermal progenitor, the pluripotent bone marrow stem cell. The coordinate transcriptional regulation of sets of lineage-specific genes is one of the important molecular mechanisms underlying hematopoietic lineage determination and differentiation. The immunoglobulin and T cell receptor (TCR) genes have been used as model systems to study lineage-specific transcriptional regulation during lymphoid development. This review summarizes our current understanding of the regulation of TCR gene expression during thymocyte ontogeny. Expression of each of the TCR genes is controlled by T cell-specific transcriptional enhancers that bind partially overlapping sets of ubiquitous and lymphoid-specific transcription factors. These include members of both the ATF/CREB family of basic-leucine zipper proteins and the Ets protooncogene family, as well as the T cell-specific zinc finger transcription factor, GATA-3, and the T cell-specific high mobility group proteins TCF-1 and TCF-1α/LEF-1. The identification of binding sites for these same transcription factors in a number of additional T cell-specific genes suggests that they may play important roles in the coordinate regulation of gene expression that specifies the development of the T cell lineages. Recent studies of the TCR α and γ genes have suggested that negative regulatory elements or transcriptional silencers may also play an

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